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# CHAPTER 41

# Bunyaviridae and Their Replication

## Part II: Replication of Bunyaviridae



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The Bunyaviridae family was established in 1975 to encompass a large group of arthropod-borne viruses sharing morphological, morphogenic, and antigenic properties (48,126,127). More than 250 serologically distinct members comprise Bunyaviridae, making it the largest known family of RNA animal viruses (16,80). Five genera of Bunyaviridae have been defined: *Bunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus*, and *Uukuvirus*. Prototype viruses for each respective genus are Bunyamwera (BUN), Hantaan (HTN), Crimean-Congo hemorrhagic fever (CCHF), sandfly fever Naples (SFN), and Uukuniemi (UUK) (14,16,142,144).

Most viruses in the family have been isolated from or are transmitted by arthropods (99,102). Hantaviruses are exceptions; these viruses are primarily rodent-borne and have no known arthropod vector but instead are transmitted *via* aerosolized rodent excreta (88). Some members of the Bunyaviridae have been associated with severe or fatal human infections; for example, Rift Valley fever virus (RVFV), HTN, CCHF, and La Crosse (LAC), but many are not known to infect humans. Readers are referred to Chapter 42 for details on the ecology, epidemiology, and medical significance of viruses in this family. Serological cross-reactivity has not been found among viruses in different genera of the Bunyaviridae. Viruses do, however, share several common structural, genetic, replicative, and morphogenic properties, which are discussed below.

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## VIRION MORPHOLOGY AND STRUCTURE

### Morphology

Morphological properties vary among viruses in each of the five Bunyaviridae genera; however, virions generally are spherical, 80–120 nm in diameter, and display surface glycoprotein projections of 5–10 nm which are embedded in a lipid bilayered envelope approximately 5 nm thick. Unique external features have been described for representatives of each genus. The surface structure of UUK is defined by clustered glycoproteins which form hollow, cylindrical morphological units (Fig. 1A). Negative staining following glutaraldehyde fixation, as well as freeze-etching, were

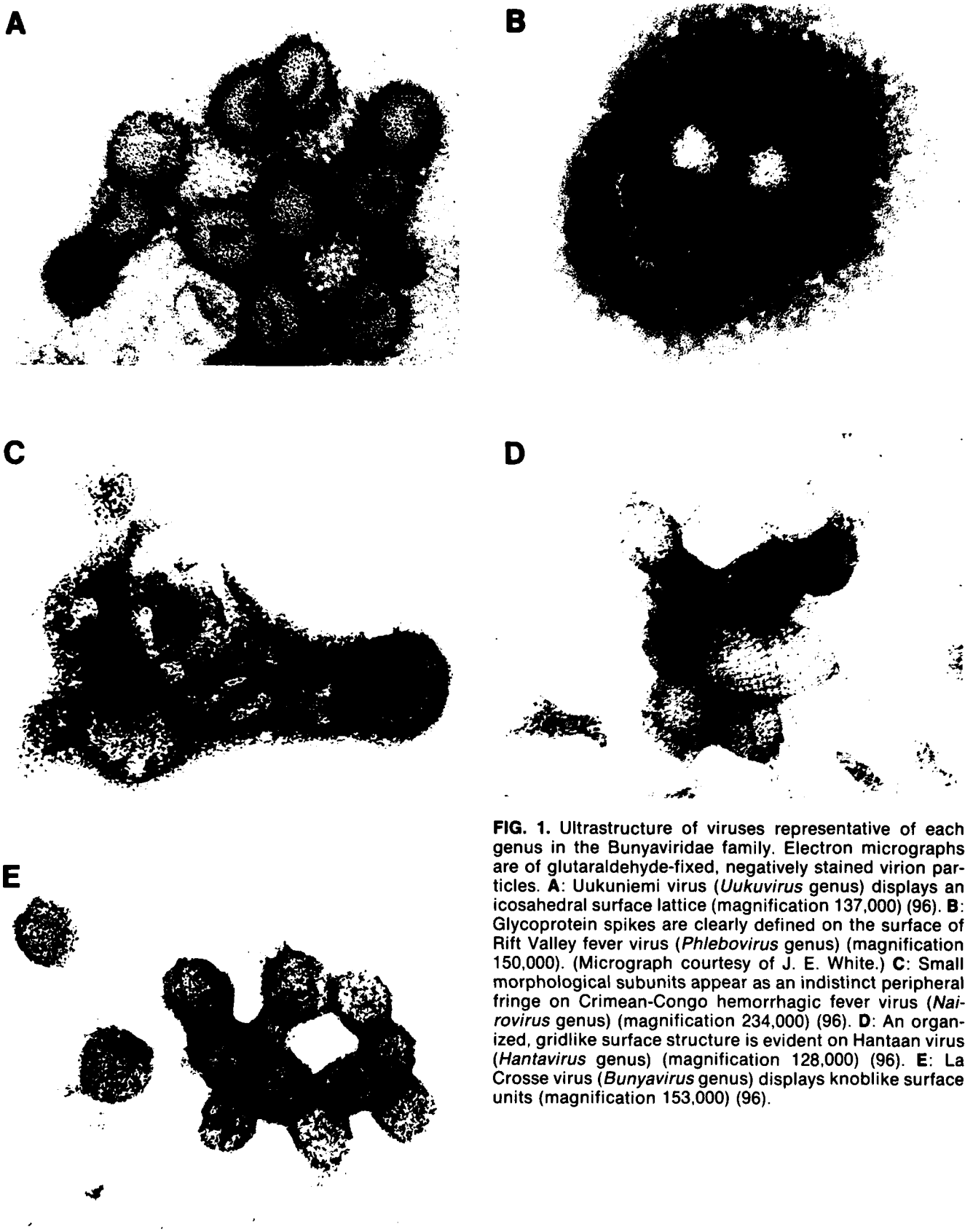


**FIG. 1.** Ultrastructure of viruses representative of each genus in the Bunyaviridae family. Electron micrographs are of glutaraldehyde-fixed, negatively stained virion particles. **A**: Uukuniemi virus (*Uukuvirus* genus) displays an icosahedral surface lattice (magnification 137,000) (96). **B**: Glycoprotein spikes are clearly defined on the surface of Rift Valley fever virus (*Phlebovirus* genus) (magnification 150,000). (Micrograph courtesy of J. E. White.) **C**: Small morphological subunits appear as an indistinct peripheral fringe on Crimean-Congo hemorrhagic fever virus (*Nairovirus* genus) (magnification 234,000) (96). **D**: An organized, gridlike surface structure is evident on Hantaan virus (*Hantavirus* genus) (magnification 128,000) (96). **E**: La Crosse virus (*Bunyavirus* genus) displays knoblike surface units (magnification 153,000) (96).

used to demonstrate that the surface units of UUK are penton–hexon clusters arranged in a T = 12, P = 3 icosahedral surface lattice with hexon–hexon distances estimated at 12.5–16 nm for stained viral particles and 17 nm for freeze-etched samples (160).

Distinct, regular, and sharply defined round surface structures about 10 nm in diameter are observed on the phleboviruses, Punta Toro (PT) (152), sandfly fever Sicilian (SFS), and RVFV (96), and the arrangement of the subunits appears to be quite similar to that described for UUK (Fig. 1B). The surface structure of the nairoviruses, CCHF (36,96) and Qalyub viruses (28), are clearly different from those of uukuviruses and phleboviruses and exhibit less distinct, small morphological surface subunits (Fig. 1C). Hantaviruses sometimes display an unusual gridlike pattern on their surfaces and elongated particles (110–210 nm long) are often observed (Fig. 1D) (71,72,96,100,165). Bunyaviruses exhibit knoblike morphological units with no distinct pattern when observed after glutaraldehyde fixation and negative staining (Fig. 1E) (96), but a lipid bilayer and well-defined surface spikes can be observed in vitrified-hydrated preparations of LAC (156).

### Structural Components

The internal organization of virion particles has been inferred from biochemical and morphological studies (see inset, Fig. 5). An overall chemical composition of 2% RNA, 58% protein, 33% lipid, and 7% carbohydrate was estimated for UUK virion particles (105). All viruses in the family have three single-stranded RNA genome segments designated as large (L), medium (M), and small (S). Consensus 3′ terminal nucleotide sequences have been found on the L, M, and S genome segments of viruses within each genus but which differ from those of viruses in other genera (except for uukuviruses and phleboviruses which have the same terminal 3′ sequences) (Table 1) (30,31,33, 108,144). Sequences complementary to those at the 3′ termini have been identified on the 5′ termini of gene segments of several viruses in the family by analysis of viral RNA (106) or cDNA clones (2,22,24,33,44,56, 57,64,74,75,89,137,145,148.) (Table 1). The complementary ends have been postulated to allow formation of stable, base-paired, pan-handle structures (Fig. 2) (33,111,137,145,148) and probably are the basis of the noncovalently closed circular RNA structures observed by electron microscopy (67). The complementary ends may also play a role in replication, possibly by serving as a transcriptase recognition structure.

The RNA segments complex with numerous copies of a nucleocapsid protein (N) to form individual L, M, and S nucleocapsids, which appear to be helical in structure (159,160). The nucleocapsids can be released by nonionic detergent disruption of virion particles and often also appear as circular structures in electron micrographs, suggesting that the complementary RNAs can base-pair even when complexed with protein in an estimated ratio of 4% RNA:96% protein (104,123,130). This assumption is supported by the ability to cross-

**TABLE 1.** *Terminal nucleotide sequences of the L, M, and S genome segments of representative members of the Bunyaviridae*

| Genus | Virus | Gene segment | 3′ terminus[a] | 5′ terminus[b] |
|---|---|---|---|---|
| *Bunyavirus* | La Crosse | S | 3′ UCAUCACAUGAGGUG | 5′ AGUAGUGUGCUCCAC |
| | | M | 3′ UCAUCACAUGAUGGU | 5′ AGUAGUGUGCUACCA |
| | | L | 3′ UCAUCACAUGAGGAU | |
| *Hantavirus* | Hantaan | S | 3′ AUCAUCAUCUGAGGG | 5′ UAGUAGUAUGCUCCC |
| | | M | 3′ AUCAUCAUCUGAGGC | 5′ UAGUAGUAGACACCG |
| | | L | 3′ AUCAUCAUCUGAGGG | |
| *Nairovirus* | Qalyub | S | 3′ AGAGAUUCUGCCUGC | |
| | | M | 3′ AGAGAUUCUUUAUGA | |
| | | L | 3′ AGAGAUUCUUUAAUU | |
| *Phlebovirus* | Rift Valley fever | S | 3′ UGUGUUUCGG | |
| | | M | 3′ UGUGUUUCUGCCACGU | 5′ ACACAAAGACCGGUG |
| | | L | 3′ UGUGUUUCUG | |
| *Uukuvirus* | Uukuniemi | S | 3′ UGUGUUUCUGGAGGUU | |
| | | M | 3′ UGUGUUUCUGCCGAUU | 5′ ACACAAAGACGGCUA |
| | | L | 3′ UGUGUUUCUGGAGUUG | |

[a] 3′ Terminal sequences were obtained by direct sequencing of virion RNAs or by analysis of cDNA clones. Sequences identical on all three genome segments are underlined.

[b] 5′ Terminal sequences were determined by sequence analysis of cDNA.

Data are from ref. 24, 30, 31, 64, 106 (La Crosse); 144, 145, 148 (Hantaan); 30 (Qalyub); 33 (Rift Valley fever virus); 108 and 137 (Uukuniemi).

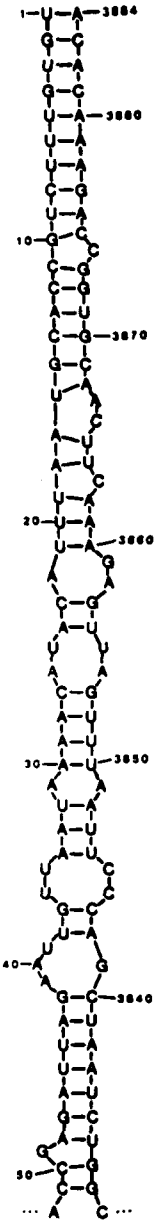

**FIG. 2.** Predicted base-pairing of the 3′ and 5′ terminal nucleotide sequences of the phlebovirus Rift Valley fever virus M segment RNA. The structure shown is energetically favored with a calculated free-energy of −38.3 kcal/mol. (From ref. 33.)

link the ends of nucleocapsid-enclosed RNAs by treatment with photoreactive, nucleic acid cross-linking agents, such as psoralens (130). Equal numbers of nucleocapsids may not always be packaged in mature virions, as evidenced by varying, nonequimolar ratios of L, M, and S RNA molecules extracted from purified virions (15). Talmon et al. (156), speculated that the size differences of virion particles observed by electron microscopy may directly relate to the number of nucleocapsids incorporated into a particular virion.

A virion-associated polymerase, which is believed to copy the negative-sense viral genome into messenger-sense RNA(s), has been described for the bunyaviruses, Lumbo and LAC (21,113), the uukuvirus, UUK (132), and the hantavirus, HTN (142). It is presumed that a large polypeptide (L) (approximately 200 kd) observed in virion preparations, is responsible for this enzymatic activity. Nothing is known about the precise location of the L protein within virions.

In contrast to members of three other negative-sense RNA virus families (Orthomyxoviridae, Paramyxoviridae, and Rhabdoviridae), viruses in the Bunyaviridae have no internal matrix proteins (see Chapters 31, 34, and 39). No enzymatic activity (other than transcriptase activity) has been found in association with any of the viral proteins.

The envelope proteins of several members of the Bunyaviridae will hemagglutinate goose erythrocytes at a characteristic (generally low) pH optimum (7,11,59). The carbohydrate moiety on the envelope proteins of all viruses in the family for which data are available has been found to be N-linked and mostly of the high-mannose rather than complex type, and no evidence for the presence of O-linked oligosaccharides has been obtained (86,87,93,120,146).

## CODING STRATEGIES OF VIRAL GENES

Coding properties of the S and/or M genome segments of viruses in the *Bunyavirus*, *Phlebovirus*, *Uukuvirus*, and *Hantavirus* genera have been described. These studies have revealed both similarities and remarkable differences in the strategies used for generation of viral proteins. Although all viruses in the Bunyaviridae family studied to date encode N in the viral complementary-sense RNA (cRNA) of their S genome segments, this appears to be the only common feature of S segment coding. The M genome segments of all viruses so far examined encode their two envelope glycoproteins, G1 and G2, in a single, continuous open-reading frame (ORF) in the cRNA. No significant coding regions have been detected in other reading frames of cRNA or vRNA; thus, a simple, negative-sense strategy is used by the M genome segments for generation of viral envelope proteins. A variety of differences, however, have been discerned in both the coding strategies and the number of proteins encoded in the M segments of viruses in the different Bunyaviridae genera. A summary of available coding strategy information is illustrated in Figs. 3 and 4.

### S Segment Strategies

The first coding strategy elucidated for any member of the Bunyaviridae was that of the S segment of the bunyavirus, snowshoe hare (SSH). Coding of the 26.5-

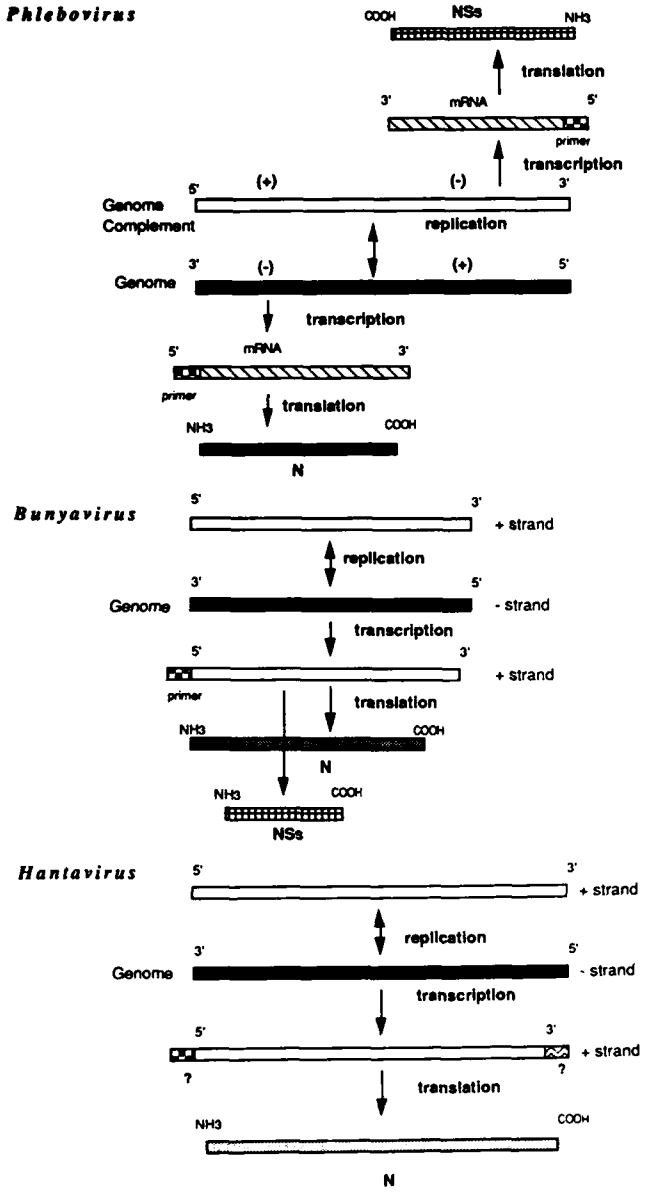


**FIG. 3.** Coding strategies of the S genome segments of viruses in the Bunyaviridae family. Unique S segment strategies have been described for viruses in the *Phlebovirus*, *Bunyavirus*, and *Hantavirus* genera. Phleboviruses utilize subgenomic messenger RNAs (mRNAs) and an ambisense strategy to encode their nucleocapsid protein (N) in the viral-complementary-sense RNA and a nonstructural ($NS_S$) protein in viral-sense RNA. Host-derived transcriptional primers are found on the 5′ termini of both subgenomic messages (73,75,107,109). Bunyaviruses encode N and $NS_S$ in overlapping reading frames of the viral-complementary-sense RNA and also utilize host-derived transcriptional primers. A single mRNA, truncated at the 3′ termini as compared to virion RNA, is believed to code for both proteins (2,3,6,17,22,24,36,39,45,112). Hantaviruses use a simple negative-sense strategy to encode N. No evidence for $NS_S$ proteins has been obtained. The 3′ and 5′ termini of hantaviral mRNAs have not been defined. No S segment coding information is available for viruses in the *Uukuvirus* or *Nairovirus* genera. In all diagrams $NH_3$ and COOH, respectively, indicate the amino and carboxy termini of translation products.

kd N protein and also a 7.4-kd nonstructural protein ($NS_S$) was localized to the viral S genome segment by *in vitro* translation of hybrid-selected messenger RNAs (mRNA) (26). These results were confirmed by analysis of polypeptides obtained from cell cultures infected with SSH and LAC reassortant viruses (i.e., viruses with various complements of L, M, or S from either SSH or LAC) (49,50,55). Similar results with less closely related viruses in the *Bunyavirus* genus identified the encoding of N in the S genome segment as a generic property (22,39). Cloning, sequence anal-

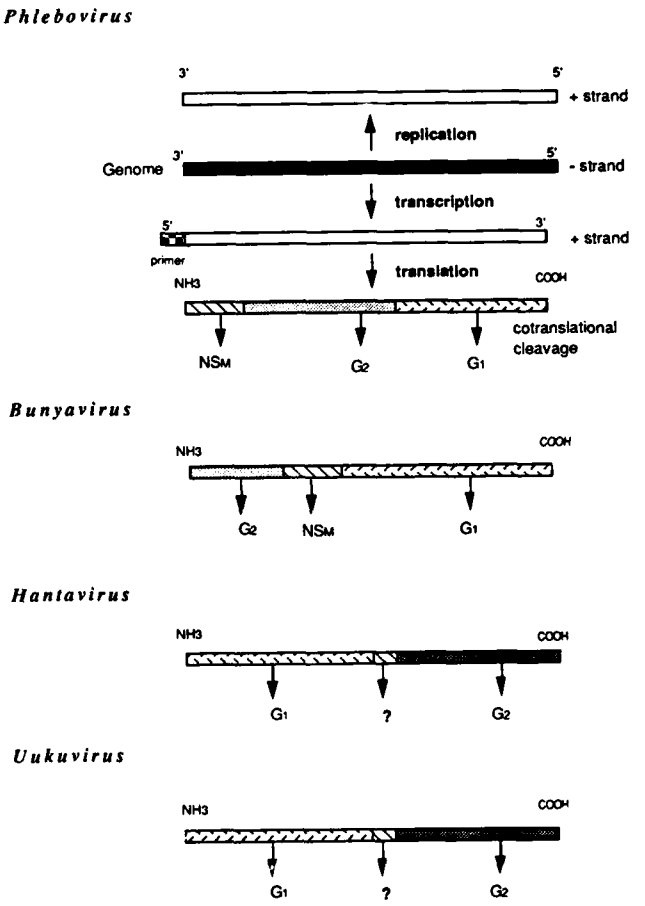


**FIG. 4.** Coding strategies of the M genome segments of viruses in the Bunyaviridae family. All viruses encode their envelope glycoproteins (G1 and G2) in a continuous open-reading frame in the viral-complementary-sense RNA. *Phlebovirus* (9,32,33,74) and *Bunyavirus* (44,47,64,89) M segments also contain coding information for nonstructural ($NS_M$) polypeptides, but those of hantaviruses and uukuviruses probably do not. A small intergenic region, however, may be removed during processing of the polyprotein precursor of G1 and G2 (137,148). Utilization of host-derived transcriptional primers has been described for phleboviruses (33) and is presumed for others. Cotranslational cleavage of the encoded polyprotein has been reported for both phleboviruses (155) and uukuviruses (137,158). No coding information is available for viruses in the *Nairovirus* genus. In all diagrams, $NH_3$ and COOH, respectively, indicate the amino and carboxy termini of translation products.

yses, and hybridization studies with cDNA representing the S genome segments of SSH, LAC, Aino, and Germiston (GER) viruses revealed that the N and $NS_S$ polypeptides are encoded in overlapping reading frames in the cRNA (2,3,17,24,56). Bunyaviruses therefore use the same nucleotide sequences, but in different reading frames, to encode two polypeptides. Only one S segment mRNA has been described for the bunyaviruses SSH, LAC, and GER (22,45,112). Thus, a single mRNA species appears to be used for translation of both N and $NS_S$.

The coding strategy of the S segments of phleboviruses was found to be even more unusual. Like the bunyaviruses, phleboviruses encode both N and $NS_S$ in their S genome segments. The phlebovirus virion S RNA is about twice the size of the bunyavirus S segment (i.e., approximately 1,900 versus 980 nucleotides) and consequently has more than sufficient potential coding information for both N and $NS_S$ without accessing overlapping reading frames. Both the 28-kd N and the 31-kd $NS_S$ proteins of the phleboviruses RVFV and Karimabad can readily be detected in lysates of infected cells (109,152). *In vitro* translation studies demonstrated that these polypeptides are generated from separate, subgenomic messages (109). Two subgenomic messages have also been identified in lysates of cells infected with the related phlebovirus PT; however, a corresponding $NS_S$ protein was difficult to discern in the same lysates (75). Cloning, sequence analysis, and cell-free translation studies revealed that the 5′ half of PT S segment cRNA contains an ORF encoding the N protein (73,107). A second ORF capable of encoding a 26-kd polypeptide was located at the 5′ end of viral-sense RNA (vRNA). This polypeptide was expressed in a baculovirus system, and antisera prepared to it could be used to precipitate small amounts of a similarly sized protein from PT-infected lysates (107). It was therefore postulated that this polypeptide is analogous to the $NS_S$ protein identified in RVFV-infected cells. This so-called "ambisense" strategy of encoding two proteins from nonoverlapping regions of virion-sense and anti-virion-sense RNAs has also been identified in the S segment of arenaviruses, which encode N and a precursor of the two viral glycoproteins in cRNA and vRNA, respectively (see Chapter 43).

Although no coding information is yet available for the S segments of viruses in the *Uukuvirus* genus, subgenomic messages and $NS_S$ proteins comparable in size to those of the phleboviruses have been identified in cells infected with UUK, suggesting that viruses in this genus have an S segment coding strategy similar or identical to that of phleboviruses (158).

Unlike bunyaviruses, phleboviruses, and uukuviruses, members of the *Hantavirus* genus apparently do not encode any $NS_S$ polypeptide. The 48-kd HTN N protein is much larger than those of viruses in the former genera yet originates from an RNA segment similar in size to that of the phleboviruses and uukuviruses. The mRNA encoding HTN N is nearly equivalent in size to genomic S RNA and no subgenomic messages were detected in infected cells (145,147). RNA transcripts of cDNA (which corresponded to Hantaan S cRNA) were able to program a cell-free translation system to yield N, indicating that the single, continuous ORF in the HTN S segment cRNA encoded N. No other significant ORFs could be detected in either cRNA or vRNA, with the possible exception of a 48 amino acid coding region, which initiated immediately after the stop codon for N and in the same reading frame (145). No polypeptides corresponding to such a 6-kd product have as yet been identified in infected cell lysates. The termini of the mRNAs have not been determined, so it is not known if the messages have truncated 3′ terminal sequences or carry host-cell sequences at their 5′ ends as do bunyaviruses and phleboviruses. The coding strategy of the S segment of hantaviruses therefore appears to be strictly negative sense with only one gene product. The significance of $NS_S$ proteins for viruses in the Bunyaviridae is not known, although they have been postulated to have some role in viral replication. Consequently, it is not possible to determine whether a function provided by the $NS_S$ products of viruses in other genera is accomplished by the much larger N of HTN, or whether hantaviruses replicate in the absence of such a function.

Complete coding properties of the S genome segments of viruses in the *Nairovirus* genus are not currently known. Both the S genome segments and the N proteins of nairoviruses are very similar in size to those of hantaviruses (29,141,145). Consequently, very little excess coding information is available if a strict negative-sense strategy such as that of Hantaan S is followed.

### M Segment Strategies

The most detailed information concerning M segment coding and expression of viral genes has been obtained with the phlebovirus RVFV. The 3,885-nucleotide RVFV M segment has a potential coding capacity of 133 kd in the major ORF of cRNA (9,33). As with the S segment mRNA, M segment mRNA has a 3′ terminal truncation of approximately 100 nucleotides as compared to vRNA (32). Amino-terminal sequence analysis of purified G1 and G2 revealed a gene order of 5′ G2-G1 3′ with respect to cRNA and indicated that a coding potential of approximately 17 kd existed between the first ATG translation initiation codon and the amino terminus of G2 (33). This pre-

glycoprotein, or nonstructural M ($NS_M$) region, has five in-frame ATG codons which precede G2, the first four of which can be used to efficiently initiate synthesis of G2 and G1. *In vitro* translation of an RNA transcript of cDNA containing all five of the initiation codons produced a primary translation product of 133 kd which could be cleaved to yield the two viral glycoproteins in the presence of microsomal membranes. The cleavage was demonstrated to occur cotranslationally as evidenced by the absence of cleavage if the microsomal membranes were added after synthesis of the precursor polypeptide (155). In addition to G1 and G2, this transcript yielded 78- and 14-kd polypeptides, corresponding in size to polypeptides previously observed in lysates of cells infected with authentic RVFV (33,78,155). Results obtained by vaccinia virus expression of cDNAs that had been altered to remove one or more of the initiation codons, and immune precipitation of expressed products by anti-peptide antibodies, demonstrated that the 78-kd protein initiates at the first ATG and the 14-kd protein at the second ATG (78,155). Similarly, the 78-kd protein was not expressed by baculovirus recombinants that had the first ATG removed; and neither the 78-kd protein nor the 14-kd protein were expressed by recombinants initiating at the fourth ATG (150). Therefore, the 78-kd protein is translated from preglycoprotein and G2 sequences starting at the first ATG, and the 14-kd protein is translated from preglycoprotein sequences starting at the second ATG. Pulse-chase experiments revealed no precursor/product relationship between the 78- and 14-kd proteins; thus, it appears that utilization of the first and second ATGs, respectively, is what dictates generation of these proteins (34). The reason why the 78-kd protein is not processed at the $NS_M$-G2 junction has not been fully ascertained. Interestingly, however, a potential N-linked glycosylation site located in the $NS_M$ region and shared by the 78- and 14-kd polypeptides was found to be utilized only in the 78-kd product. Removal of the glycosylation signal by site-directed mutagenesis appeared to have no effect on production of the 14- and 78-kd proteins, suggesting that glycosylation alone is not what prevents cleavage in the 78-kd protein at the $NS_M$-G2 junction. It therefore appears that translation initiation at the first ATG (and consequently the presence of the 37 amino acids located between the first and the second ATGs) results in utilization of the glycosylation site and the lack of cleavage at the junction of the preglycoprotein region and G2 (34,79). The significance of these $NS_M$ polypeptides is not known; however, the apparent routine use of two in-frame initiation codons, both *in vivo* and *in vitro*, to regulate posttranslational protein modification suggests that they may serve some function.

Complete sequence information for the M genome segment of another member of the *Phlebovirus* genus, PT, has also been obtained (74). The M segment of PT is larger than that of RVFV (4,330 nucleotides) and has a larger (30 kd) potential preglycoprotein coding region, although no $NS_M$ protein(s) analogous to those of RVFV has been detected in PT-infected cells. The gene order is reversed (i.e., 5′ G1-G2 3′), as compared to that of RVFV; however, this probably has no biological significance because the G1 and G2 designations refer only to relative mobilities on polyacrylamide gels and do not correlate with functional properties of the proteins. This is clearly illustrated by computer-predicted homologies indicating that RVFV G1 is related to PT G2 and that RVFV G2 is related to PT G1 (74). No homology was detected between predicted $NS_M$ products. Like RVFV, it was found that PT G1 and G2 could be efficiently expressed by vaccinia virus recombinants in the absence of $NS_M$ coding sequences (98). Thirteen in-frame initiation codons are located in the preglycoprotein coding sequences of the PT M segment, but no *in vivo* or *in vitro* data are yet available concerning their usage. Three potential glycosylation sites reside in the preglycoprotein coding information of PT (74). It will be interesting to determine whether a selective use of glycosylation sites similar to that observed in RVFV is employed in PT $NS_M$ gene product(s).

Carboxy-terminal sequence information has not been obtained for phlebovirus envelope proteins; consequently, the exact point of cleavage between G1 and G2 has not been identified. However, an intergenic region of less than 27 amino acids was demonstrated between G2 and G1 coding information of RVFV by immune precipitation of viral proteins with antisera to synthetic peptides representing amino acid sequences at $-16$ to $-27$ upstream of the amino terminus of G1 (78).

Although serological cross-reactivity between uukuviruses and phleboviruses has not been demonstrated, viruses in these genera have several genetic features that suggest an evolutionary relationship. Terminal complementary 3′ and 5′ sequences of the M segments of UUK are identical for 10–15 nucleotides to those of RVFV and PT (Table 1) (33,108,137). Complete sequence analysis of the M genome segment of UUK, however, revealed a coding strategy different from that of phleboviruses. The UUK M genome segment consists of 3,231 nucleotides and, like those of the phleboviruses, encodes the G1 and G2 proteins (both 55 kd) in a single ORF of the cRNA. The envelope proteins are also processed by cotranslational cleavage of a polyprotein precursor (137,158). Unlike the phleboviruses, uukuviruses have no preglycoprotein coding region and the amino terminus of G1 is located 17 amino acids downstream of the first (and only) initiation codon. Thus, UUK G1 appears similar to the RVFV 78-kd protein. A possible intergenic region

of 81 amino acids was found between the predicted transmembrane, carboxy-terminal anchor of G1 and the putative signal sequence of G2. Dot matrix comparison of the predicted G1 and G2 amino acid sequences of UUK, RVFV, and PT revealed an obvious but low degree of homology among polypeptides encoded in the 5′ portion of the cRNAs of each virus and also among those encoded in the 3′ portion of the cRNAs. No homology with the $NS_M$ coding regions of either PT or RVFV could be identified (137). Because few or none of the polypeptides encoded in the preglycoprotein region are detected in virion preparations of RVFV or PT (although a small amount of 78-kd polypeptide is found in RVFV virion preparations), these polypeptides must be cleaved proteolytically prior to extrusion of mature virus particles (33,74). Until a function can be assigned to these $NS_M$ polypeptides, it is impossible to determine whether UUK replicates in the absence of such a function or accomplishes whatever function is required without removal of a portion of the amino terminus of G1.

Like UUK, the hantavirus HTN encodes G1 and G2 in a continuous ORF in the cRNA of the M genome segment and does not appear to encode $NS_M$ polypeptides. The M genome segment of HTN consists of 3,616 nucleotides and has a short, 17 amino acid leader sequence between the first initiation codon and the amino terminus of G1 (148). A second in-frame initiation codon, located nine amino acids upstream of the amino terminus of G1, apparently is not used, as indicated by the inability to express G1 and G2 in vaccinia or baculovirus systems if the first codon is not present (151). The carboxy terminus of Hantaan G1 was localized to within 34 amino acids of the amino terminus of G2 by immune precipitation of viral proteins with antisera prepared to synthetic peptides representing M segment sequences. It was not determined if the 34 amino acids represented an intergenic region; however, at least 19 of these amino acids have characteristics that suggest they constitute a signal sequence for G2 (148). Immune precipitation with antipeptide antibodies was also used to determine that the carboxy terminus of G2 extends to the end of the coding information of M segment cRNA. No other significant ORFs were detected in either the vRNA or cRNA of HTN M. Consequently, with the possible exception of the potential intergenic coding region, no coding information for a $NS_M$ polypeptide is available. The envelope glycoproteins of both hantaviruses and uukuviruses therefore appear to be the only gene products of the viral M segments. No sequence homology can be detected between the M genome segment of HTN and those of PT, RVFV, or UUK.

Nucleotide sequence analyses of cDNA representing the M genome segments of the bunyaviruses BUN, SSH, and LAC also revealed a continuous ORF in the cRNA (44,47,64,89). The gene order within the 4,527-nucleotide M segment of SSH was determined by amino- and carboxy-terminal sequencing of G1 and G2 and was found to be 5′ G2-G1 3′ (44,47). A coding potential of 19 kd of protein was discovered between G2 and G1 coding sequences, which is more than sufficient to encode the 11-kd $NS_M$ polypeptide previously identified in authentic SSH-infected cell lysates (47,49). Antisera to synthetic peptides representing amino acids predicted from the intergenic coding region were able to immune precipitate 10- and 11-kd polypeptides from SSH-infected cell lysates, thus supporting the conclusion that $NS_M$ proteins are encoded in that region. These putative $NS_M$ proteins have not yet been analyzed functionally.

No information is currently available concerning the M segment coding strategy of nairoviruses.

### L Segment Strategies

Very little is known about the coding properties of L segments of viruses in the Bunyaviridae. The L proteins of two bunyaviruses, LAC and Tahyna, were found to have different electrophoretic migrations on acrylamide gels. With this method to differentiate reassortant viruses containing the L segments of either LAC or Tahyna, the L genome segments were demonstrated to encode the L proteins of those viruses (43). The L protein presumably functions as the viral transcriptase; however, definitive data to that effect have not yet been obtained. Elucidation of both the expression strategy of the L genome segment and the functional properties of the gene product(s) awaits molecular cloning and sequence analysis.

## STAGES OF REPLICATION

The principal stages of the replication process for viruses in the Bunyaviridae are illustrated in Fig. 5 and can be summarized as follows:

1. Attachment, mediated via an interaction of viral proteins and host receptors.
2. Entry and uncoating, probably by endocytosis of virions and fusion of viral membranes with endosomal membranes.
3. Primary transcription: that is, the synthesis of viral-complementary mRNA species with genome templates, host-cell-derived primers, and the virion-associated polymerase.
4. Translation of primary L and S segment mRNAs by free ribosomes, translation of M segment mRNAs by membrane-bound ribosomes, and primary glycosylation of nascent envelope proteins.

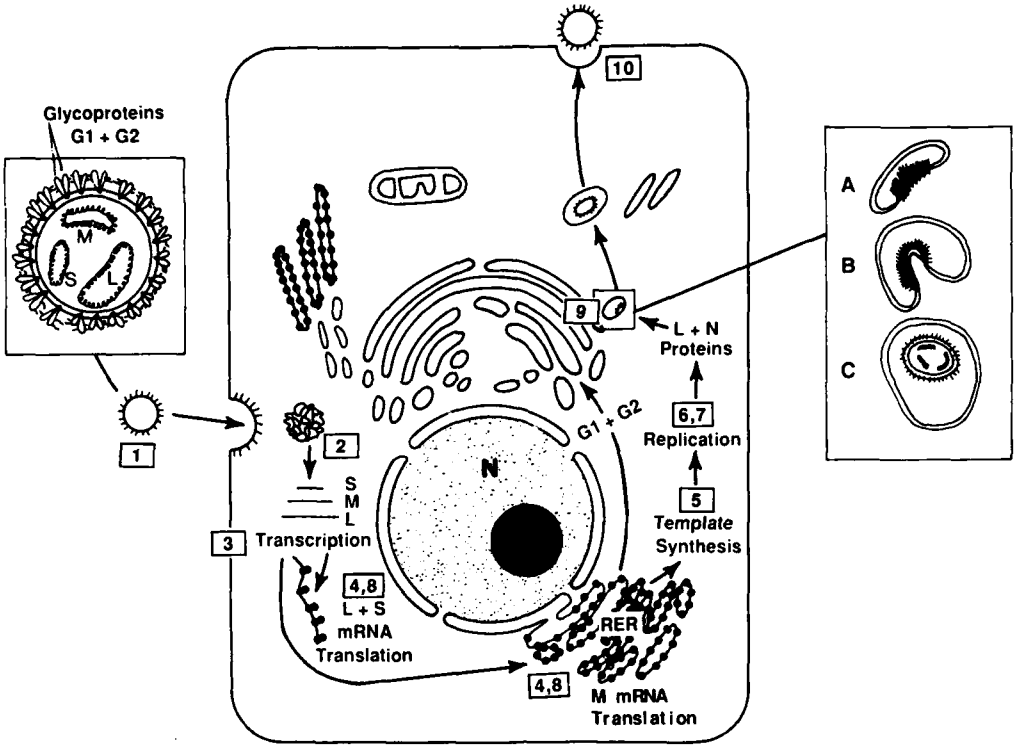


**FIG. 5.** Summary of probable replication processes for viruses in the Bunyaviridae. Virion particles (inset on left) contain large (L), medium (M), and small (S) viral RNAs complexed with nucleocapsid proteins. The bilaminar, lipid envelope has integral virus-specified glycoproteins (G1 and G2) which interact across the membrane with the ribonucleoprotein structures. The precise location of the virion-associated polymerase is not known. Numbered events corresponding to those listed in the text include: (1) attachment; (2) entry and uncoating; (3) primary transcription to yield viral messenger RNAs (mRNA); (4) translation of L and S segment mRNA on free ribosomes: translation M segment mRNA on membrane-bound ribosomes and primary glycosylation of the gene products (G1 and G2); (5) synthesis of anti-genome templates; (6) genome replication; (7) secondary transcription; (8) further translation; (9) terminal glycosylation of G1 and G2 and assembly of viral particles by budding into Golgi vesicles; (10) transport of cytoplasmic vesicles to the cell surface, fusion and release of mature virions. The stages of budding into smooth membrane vesicles (inset on right) are: (A) ribonucleoprotein structures accumulate on the cytoplasmic face of membranes which have G1 and G2 embedded into them and exposed on the luminal side; (B) Involution of membranes; (C) completion of budding to yield a morphologically mature virion within a cytoplasmic vacuole (152). Abbreviations for cellular substructures are: N, nucleus; RER, rough endoplasmic reticulum.

5. Synthesis and encapsidation of viral-complementary RNA to serve as templates for genomic RNA or, in some cases, for subgenomic mRNA.
6. Genome replication.
7. Secondary transcription: that is, the amplified synthesis of the mRNA species and ambisense transcription.
8. Continued translation and RNA replication.
9. Morphogenesis, including accumulation of G1 and G2 in the Golgi, terminal glycosylation, and acquisition of modified host membranes, generally by budding into the Golgi cisternae.
10. Fusion of cytoplasmic vesicles with the plasma membrane and release of mature virions.

### Attachment and Entry

The early events in the infection process of members of the Bunyaviridae are not well defined. Like other enveloped viruses, one or both of the integral viral envelope proteins mediate attachment to host-cell receptors. This was first demonstrated by proteolytic enzyme treatment of purified LAC, which resulted in "spikeless" virus particles, and a five-log reduction in infectivity as compared to nondigested virions (104). The nature of cell receptors involved in attachment has not been identified thus far for any member of the family. The viral proteins involved in attachment, however, have been examined indirectly by using polyclo-

nal and monoclonal antibodies to block infection or hemagglutinating activity. For example, neutralization of LAC can be obtained with antibodies directed against G1, indicating a relationship of that protein to infectivity (59,63,82,83). Although monoclonal antibodies to LAC G2 have not been examined, it was suggested that LAC G1 is more actively involved in binding to host cells than is G2, based on the finding that treatment of LAC with bromelain or pronase, which degrade portions of G1 but leave G2 uncleaved, renders the virus completely noninfectious (82). In contrast, neutralizing and hemagglutination inhibiting (HI) sites have been detected on both the G1 and G2 proteins of HTN (7,35), RVFV (81), and PT (125), suggesting that both proteins may be involved in attachment, either directly or by conformational requirements.

Fusion of infected cells at acidic pH values has been reported for viruses in the Bunyaviridae (6,60–62) as well as numerous other enveloped viruses (46,69,90,95,97). The pH-dependent fusion is generally believed to relate to early events in the infection process, particularly the translocation of RNA and proteins into cell cytoplasm. Electron microscopy of the infection process of RVFV revealed that viral particles appeared to enter cells in phagocytic vacuoles (41). This observation is consistent with a mode of entry similar to that first described for alphaviruses in which the virus is endocytosed via coated vesicles (95). These endosomes subsequently become acidified (157), triggering a fusion of viral membranes and endosomal membranes, which results in the release of the nucleocapsid into the cell cytoplasm. Direct evidence for this process with viruses in the Bunyaviridae has not yet been obtained. Furthermore, whether one or both of the envelope proteins are necessary for fusion has not been defined for most members of the family; however, selection of a viral mutant with a defective fusion function suggested that the G1 protein of LAC mediates such fusion (61).

### Transcription

After uncoating of viral genomes, transcription of negative-sense vRNA to complementary mRNA is believed to occur by interaction of the virion-associated polymerase and the three viral RNA templates (21,132). The mechanism by which viruses in the Bunyaviridae family initiate transcription of their mRNA is less well defined but, at least for bunyaviruses and phleboviruses, displays some remarkable similarities to that of influenza viruses (see Chapter 39). Influenza viruses have been found to cleave capped and methylated oligonucleotides from host-cell mRNAs and to use them to prime transcription of viral genomes. Like those of influenza, mRNAs of both bunyaviruses and phleboviruses possess 5′ terminal extensions of approximately 15 nucleotides that are heterogeneous in sequence and are not templated from vRNA (18,19,32,113). These extensions are probably analogous to the primers acquired by influenza virus, although direct evidence for capped, methylated structures on the 5′ termini of Bunyaviridae members has not yet been demonstrated. Indirect evidence has been obtained with LAC, however, which indicates that the primers are similar to those used in influenza viral transcription. The RNA polymerase activity associated with LAC virions was found to be stimulated by oligonucleotides such as (A)nG, cap analogs such as $m^7GpppAm$, and alfalfa mosaic virus (ALMV) 4 RNA (113). As with influenza, (A)nG stimulation of LAC transcription was related to incorporation of these compounds into nascent transcripts, while that of the cap analogs was by a different, unknown mechanism. The ALMV 4 RNA was determined to stimulate LAC transcription by providing transcriptional primers which are specifically cleaved 10–14 nucleotides downstream from the cap group by a LAC virion-associated endonuclease. For this endonuclease activity to be functional, there must be at least one methyl group on the cap. These capped and methylated fragments are presumably the same as those used for priming LAC transcription, as suggested by the addition of 10–14 nucleotide extensions to LAC transcripts when ALMV 4 RNAs were used as a primer source (113).

As well as similarities, distinct differences in primary transcription by influenza virus and bunyaviruses have been identified. A nuclear step is obligate for transcription of influenza genomes due to the requirement for primers cleaved from precursors of cellular RNA. Both heterogeneous nuclear RNA (hnRNA) and mRNA transcribed by RNA polymerase II in the cell nucleus are possible candidates for these primers (76). The nuclear step is probably also prerequisite for splicing the viral mRNAs. Unlike influenza, bunyaviruses do not have spliced mRNAs and appear to replicate solely in the cytoplasm. Goldman et al. (58) reported that the bunyavirus, California encephalitis virus, could produce progeny in enucleated cells. Although LAC was found not to replicate in enucleated cells (118), other evidence suggests that LAC replication occurs exclusively in the cytoplasm. Pulse-labeling infected cells at various times after infection and examination of both cytoplasmic and nuclear fractions for labeled mRNA revealed LAC S genome transcription only in the cytoplasm (138). Moreover, unlike influenza virus, LAC has been found to be resistant to the effects of actinomycin D, a drug that inhibits DNA-dependent, RNA polymerases, such as host-cell polymerase II (105). It is believed therefore that LAC acquires the primers needed for transcription from a

stable pool of host-cell messages rather than from newly synthesized nuclear transcripts and consequently has no requirement for ongoing host RNA synthesis as does influenza.

Inhibitors of host-cell protein synthesis, such as cycloheximide and puromycin, have been found to have no effect on primary transcription (i.e., copying of viral genomes to cRNA by the virion-associated polymerase) in other negative-strand RNA virus families such as the Orthomyxoviridae (10), Rhabdoviridae (94), and Paramyxoviridae (135). Conflicting data concerning the requirement for ongoing host protein synthesis and primary transcription of viruses in the *Bunyavirus* genus have been reported. Abraham and Pattnaik (1) first reported cycloheximide sensitivity in both BUN and Akabane viruses. Similarly, either no or greatly reduced amounts of S segment mRNA could be detected in LAC-infected cell cultures by hybridization with probes complementary to the 3′ end of mRNA, if cells were treated with cycloheximide or puromycin (112,128,129). Also with LAC, the virion-associated polymerase was found to produce only incomplete transcripts *in vitro* unless rabbit reticulocyte lysates were added to provide a coupled transcription–translation system. In the coupled system, drugs that inhibit protein synthesis also inhibited full-length mRNA synthesis and resulted in the reappearance of the incomplete transcripts (12,129). This led to a hypothesis that translation of the nascent bunyavirus S segment mRNA is required to prevent premature termination of primary transcription products and that the nascent chain may be interacting with its template to cause premature termination. Such a translation requirement would be similar to that observed in certain bacterial systems, whereby ribosomes prevent RNA–RNA interaction and thus premature termination (129).

In contrast, although GER S segment mRNA was inhibited in cell culture by either anisomycin or cycloheximide, full-length S transcripts could be obtained in an *in vitro* transcription system without added translational capabilities (57). What factors are therefore responsible for the required cellular protein synthesis could not be ascertained.

Still different results were obtained with the bunyavirus, SSH, in that strand-specific cDNA probes were able to detect full-length S segment mRNA (but not vRNA) readily in the presence of puromycin or cycloheximide (45). These results suggest that SSH primary transcription is not dependent on ongoing host protein synthesis. At present, it is unclear whether the variations in transcriptional requirements for viruses in this genus are due to host-cell factors (131), differences in their transcriptional properties, or differences in the sensitivities of methods used. Elucidation of primary transcription processes of the M and L segments of bunyaviruses, as well as that of viruses in the other genera, should help clarify this issue. A definitive answer, however, will most likely await description of the virion-associated polymerase, perhaps from molecularly cloned and expressed viral L segments.

Differences between bunyavirus vRNA and mRNA are found, not only at their 5′ termini but also at their 3′ termini. The S segment mRNAs of LAC (112), SSH (26), UUK (124), Akabane (114), and GER (22) and the M segment mRNA of SSH (45) all have been shown to be truncated at their 3′ termini by about 100 nucleotides as compared to vRNA. Potential transcription termination sites have been proposed for the S segments of LAC (112) and SSH (44,45) at or near the genomic sequence, 3′-G/CUUUUU, which is similar to other negative-strand RNA viral transcription termination–polyadenylation signals (65,134) Whether this site templates polyadenylation is not known; however, the low affinity of bunyavirus mRNAs for oligo dT columns would suggest that the mRNAs are not polyadenylated extensively (114,124,158). The M segment mRNA of SSH, while shorter than that of vRNA, has no analogous 3′-CUUUUU template sequence and therefore apparently does not use a homopolymeric $U_5$ or $U_6$ tract for termination of transcription (44). Although no obvious transcription termination signal has been identified for the bunyavirus M segment mRNA, a purine-rich sequence located immediately after the putative transcription termination signal of SSH S segment mRNA and a similar sequence observed near the expected 3′ end of the M segment mRNA have been suggested to play some role in transcription termination (44,45).

For phleboviruses, transcription of the S segment is complicated by the ambisense coding strategy (see above). Both the N and $NS_S$ subgenomic mRNAs have heterogeneous, nonviral, 5′ terminal sequences and probably employ cellular primers for transcription, as do bunyaviruses (75). Hybridization studies confirmed earlier predictions based on sequence analysis that the $NS_S$ message was the same polarity as vRNA and the N message was complementary to vRNA (73–75). In the presence of the protein synthesis inhibitors, puromycin or cycloheximide, PT N mRNA was still detected, but $NS_S$ mRNA was not. These results are consistent with a model in which replication of full-length, encapsidated, viral-complementary S RNA must occur prior to synthesis of the $NS_S$ mRNA. This suggests that the $NS_S$ protein is not involved in the early stages of replication (e.g., primary transcription).

The transcription termination sites for both the N and $NS_S$ mRNAs of PT were mapped by hybridizing a series of synthetic oligonucleotides corresponding to viral or viral-complementary-sense sequences to the messages. The results indicated that the 3′ termini of both mRNAs were within 40 nucleotides of one another (42). Computer analysis of the intergenic region

and the sequences encoding the 3′ termini of the messages revealed a long, inverted complementary sequence which could potentially form a hairpin structure. Similar (but shorter), energetically favored structures have been identified in intergenic regions of the S segments of the arenaviruses, Pichinde and lymphocytic choriomeningitis viruses, which also use an ambisense coding strategy (136). Such structures have also been observed in other eukaryotic systems and have been postulated to be involved in transcription termination (13). Although it is believed that transcription of phlebovirus RNA, like that of bunyavirus RNA, occurs exclusively in the cytoplasm, intranuclear inclusions of a RVFV nonstructural protein (probably $NS_S$) have been found in infected cells (153).

The transcriptional properties so far identified for phlebovirus M genome segments are very similar to those of bunyaviruses. The M segment mRNA of RVFV was found to have 12–14 heterogeneous nucleotides at the 5′ end and to be truncated by approximately 112 nucleotides at the 3′ end as compared to vRNA (32). A potential purine-rich transcription termination site (nucleotides 3,761–3,774; 5′-UGGGGUGGUGGGGU), located near the mapped 3′ terminus of the RVFV M segment mRNA, was suggested, based on its similarity to sequences in analogous regions of the S RNA of SSH (nucleotides 903–914; 5′-GGUGGGGGGUGGGG), the M RNA of SSH (nucleotides 4,454–4,467; 5′-GGUGGGGGGUG GGG), and the M RNA of PT (nucleotides 4,255–4,271; 5′-GGUGAGAGUGUAGAAAG). If these purine-rich sequences are involved in transcription termination, the mechanism by which they are recognized will require further study.

Little is known about the primary transcriptional properties of viruses in the *Hantavirus*, *Uukuvirus*, and especially the *Nairovirus* genera. A manganese-dependent, virion-associated polymerase was found to produce UUK transcripts capable of hybridizing to viral RNA templates, although the transcripts appeared to be small (132). A polymerase with similar divalent cation requirements was found in association with HTN virions (142).

## Genome Replication

In negative-strand viruses, the change from primary transcription to replication requires a switch from mRNA synthesis to synthesis of full-length cRNA templates and then vRNA. The processes involved in making that switch have not been defined for any member of the Bunyaviridae family. Presumably, some viral or host factor is required to signal a suppression of the transcription termination signal responsible for generation of truncated mRNA and also to prevent the addition of the capped and methylated structures to the 5′ termini of the cRNAs. There is no question that genome replication and subsequent secondary transcription are prevented by translational inhibitors such as cycloheximide. These results indicate that continuous protein synthesis is required for replication of the genome. While it is not known which proteins are required, they are likely to be of viral origin.

For the rhabdovirus, vesicular stomatitis virus, the switch to antigenome synthesis appears to be controlled by the N protein (8,20,115,116,164). Encapsidation by N seems to serve as an antitermination signal, thus allowing full-length genome synthesis. It has been suggested that the VSV NS protein is also involved and acts to control the availability of N (68). A similar mechanism appears plausible for the Bunyaviridae, whereby N would function to regulate replication and would be tempered by the presence of nonstructural proteins where they exist. The factors dictating that vRNA and cRNA should complex with N to form nucleocapsid structures, while mRNAs should not, are not known, but it has been suggested that the added (presumably capped) host-cell sequences on the 5′ ends of viral messages may somehow prevent encapsidation (129). How N would function in orchestrating the switch from mRNA to cRNA synthesis and what function nonstructural proteins may play in the process are subjects for future research.

## Morphogenesis

### *Synthesis and Processing Viral Proteins*

The Bunyaviridae viral messenger RNAs are apparently not polyadenylated at their 3′ termini but do have modified 5′ termini in the form of short host-derived nucleotide sequences and probably cap structures (See Transcription section above). Viral polypeptides are synthesized shortly after infection, suggesting that mRNAs are transcribed and translated rapidly. Time-course studies of the synthesis of viral polypeptides for the phlebovirus RVFV revealed that, at a high multiplicity of infection (moi), radiolabeled N and $NS_S$ proteins can both be detected as early as 2 hr after infection and the envelope glycoproteins shortly thereafter (109). Consequently, if the ambisense strategy described above is correct, that is, transcription of both cRNA and vRNA is required prior to synthesis of $NS_S$ mRNA, then these events must occur very quickly. Similar kinetics of protein synthesis were observed in cell cultures infected with the bunyaviruses BUN and Trivittatus (117), and LAC for which protein synthesis was found to reach a maximum at 3–5 hr after infection (93). Uukuviruses (158) and han-

taviruses (143) exhibit a somewhat slower rate of protein synthesis in that viral proteins could not be detected until approximately 6 hr after infection. The nairovirus Dugbe also demonstrated slower kinetics of synthesis; however, only low moi studies were performed (27).

As described above, the two proteins encoded by the S segments of bunyaviruses, phleboviruses, and probably uukuviruses do not originate from precursor polypeptides, and therefore processing of proteins is not required. Hantaviruses and probably nairoviruses encode only one product in their S segment. Posttranslational modifications (e.g., phosphorylation or amidation) in general have not been defined for S segment products except for the $NS_S$ of RVFV, which appears to be phosphorylated (109).

In contrast, the M segment gene products are both processed and modified. Both G1 and G2 (and, in some genera, a nonstructural protein) are translated from a single M segment mRNA species as a polyprotein precursor and are cotranslationally cleaved. Examination of the predicted amino acid sequence of the polyprotein precursors of RVFV, PT, UUK, SSH, LAC, and HTN indicates that distinct hydrophobic regions precede both G1 and G2 and appear to represent signal sequences. This suggests that the second protein encoded in the polyprotein precursor has its own signal sequence analogous to the internal signal sequence described for the E1 protein of the alphaviruses, Semliki Forest virus (54,66), and Sindbis virus (133). Although *in vivo* mechanisms of translational initiation of the second protein of the polyprotein are not known, results of *in vitro* studies suggest an independent initiation mechanism. This was demonstrated with RVFV by deleting initiation codons preceding the amino terminus of G2 (the first encoded protein). Under these conditions, no G2 was detected in cell-free systems; however, G1 was still synthesized, indicating that an inframe ATG codon somewhere upstream of the amino terminus of G1 functioned as a translation initiator (155). Similarly, Hantaan G2 (the second protein encoded in the M segment) was expressed efficiently by vaccinia and baculovirus recombinants prepared from cDNA representing only HTN G2 sequences plus 32 upstream amino acids. The only possible initiation codon was a natural, in-frame ATG located 17 amino acids upstream from the amino terminus of G2 (151). Whether such independent initiation of the envelope proteins of members of the Bunyaviridae can or does occur in authentic viral infections is not yet known.

Also inferred from the predicted amino acid sequences are hydrophobic, carboxy-terminal anchor regions on the proteins of RVFV, HTN, PT, and UUK viruses. A common property of all M segment gene products predicted from cDNA sequences studied so far is their high cysteine content (5–7%) and, in related viruses, the conservation of the position of the cysteine residues (47,74,137,149). These findings suggest that extensive disulfide-bridge formation may occur and that the positions may be crucial for determining correct polypeptide folding.

### *Glycosylation*

All the envelope proteins examined to date possess N-linked oligosaccharides. The gene sequences that define a potential N-linked glycosylation site are those that encode Asn-X-Ser/Thr (see ref. 70). Five such sites were identified in the glycoprotein coding sequences of RVFV (33), PT (74), SSH (2,47), and LAC (64); seven sites in HTN (148); and eight in UUK (137). The number of sites actually used in mature virion proteins has not been defined for all viruses; however, G2 of the phlebovirus RVFV, was demonstrated to be glycosylated at its single available site and at least three of four possible G1 sites (79). Analysis of the predicted amino acid sequences of the envelope proteins of SSH (44,47) and examination of glycosylated tryptic oligopeptides (161) suggest that all three potential glycosylation sites on G2 are used and at least one of two sites on G1.

Two broad classes of asparagine-linked oligosaccharides, complex or high-mannose (simple), generally are found on mature glycoproteins (reviewed in ref. 91). Often both types are attached to the same polypeptide chain. As described for the hemagglutinin protein of influenza virus, as well as other glycoproteins (reviewed in refs. 84 and 85), in order for oligosaccharides to evolve from the high-mannose type to the complex type, they are normally transported through the Golgi, where mannose residues are trimmed and terminal residues added. Examination of the oligosaccharides attached to the G1 and G2 proteins of UUK in infected cells revealed that G2 has mostly high-mannose glycans, whereas G1 contains both complex and a novel intermediate-type oligosaccharide (120). Similar results were obtained with Inkoo and LAC (93,120), while the glycoproteins of HTN were found to be mostly of the high-mannose type (146).

The type and amount of oligosaccharides attached to viral proteins therefore correlate to some extent with the mode of viral maturation. For example, shortly after primary glycosylation of nascent proteins at the rough endoplasmic reticulum (ER), oligosaccharides are susceptible to cleavage by endoglycosidase H (endo-H), an enzyme that cleaves only high-mannose residues. Later, after removal of glucose residues at the rough ER, migration of the glycoproteins to the smooth ER and Golgi, trimming of residues, and attachment of peripheral sugars, the oligosaccharides are no longer susceptible to endo-H cleavage. This ac-

quired resistance to endo-H therefore generally indicates that the proteins have been processed through the Golgi. The endo-H susceptibility of the high-mannose and intermediate-type glycans found on the envelope proteins of viruses in the Bunyaviridae suggests that they are incompletely processed through the Golgi, a finding that may relate to their unusual mode of morphogenesis. It was postulated originally that vacuolization of the Golgi—which accompanies Bunyaviridae infection of cells—might prevent further processing of viral proteins by that organelle (86,87). This appears not to be the case, because both viral and nonviral glycoproteins are still efficiently transported through the Golgi, despite vacuolization in response to infection with UUK (51–53). More information is required to determine the exact nature of Golgi-associated processing events.

### *Transport*

One of the earliest notable features found to distinguish members of the Bunyaviridae from all other negative-strand RNA uses was that the viral particles are formed intracellularly by a budding process at smooth-surface vesicles in the Golgi area (Fig. 6) (15,92,101,159). It is not known what signals are responsible for the accumulation of viral structural proteins in the Golgi; however, possibilities include a structural or molecular resemblance between G1 and/or G2 and Golgi-specific proteins, such as the glycosyltransferases that are normally retained in the Golgi (51), or that they lack the signal required for transport from the Golgi to the plasma membrane (86). Whatever the reason, it is clear that the envelope proteins possess signals necessary to localize to the Golgi without

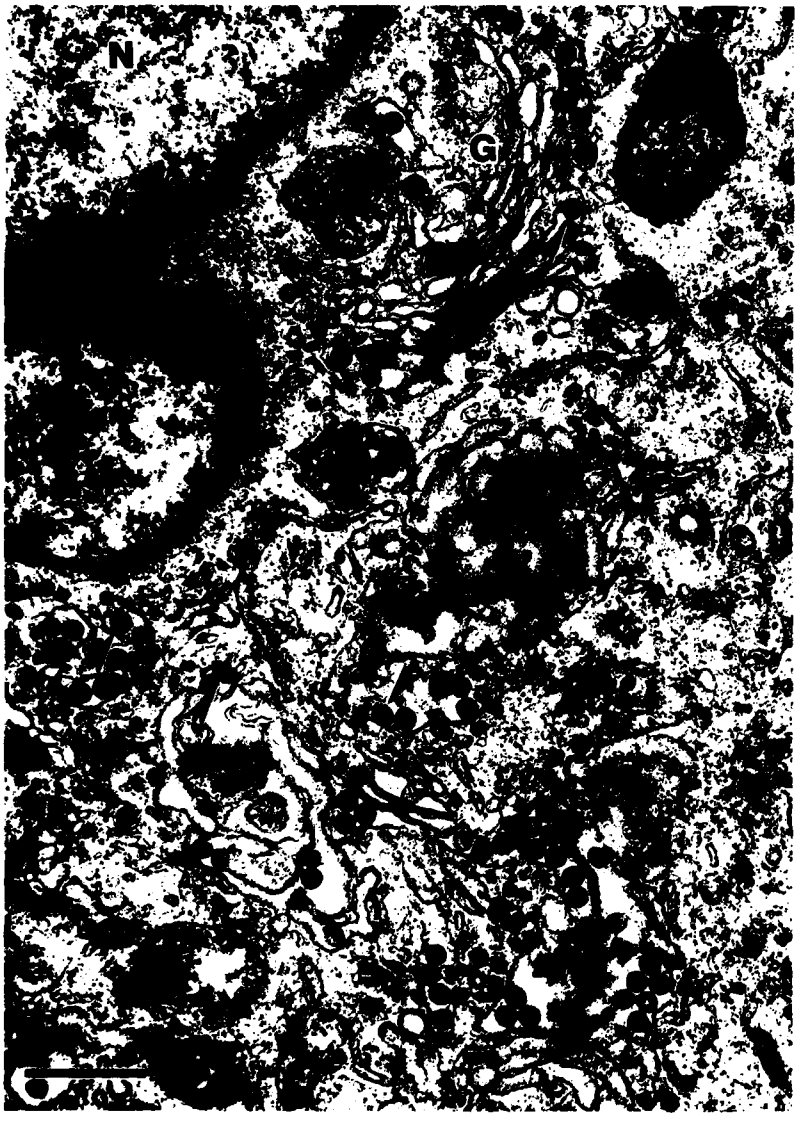


**FIG. 6.** Electron micrograph of a thin section of a RVF-infected rat hepatocyte showing budding at internal smooth membranes including Golgi cisternae. The field contains several particles in the process of budding (*arrowheads*) as well as mature virions. G, Golgi; N, nucleus. Bar represents 600 nm. (From ref. 4.)

additional requirements for other viral proteins or nucleic acid. This has been demonstrated by following the transport of vaccinia virus-expressed G1 and G2 of RVFV (162), PT (98), or HTN (119) in infected cell cultures. With RVFV, it was determined that the Golgi association occurred whether or not the preglycoprotein ($NS_M$) region was expressed in conjunction with G1 and G2 or if those sequences were removed prior to expression. The signals for Golgi localization therefore reside within G1 and G2 of RVFV virus (162).

Similar conclusions were drawn from studies with temperature-sensitive mutants of UUK, in which G1 and G2 (but not N) localized to the Golgi, despite the absence of virus maturation. These same studies demonstrated that the transport of viral envelope proteins from their site of synthesis on the rough ER through the Golgi occurred at an estimated two to three times slower rate than that of most viral membrane glycoproteins destined to be transported to the plasma membrane (51).

### *Assembly and Release*

Electron microscopy of phleboviruses (152) and UUK (86) revealed maturation mostly, but not exclusively, in perinuclear regions in association with smooth membranes, presumed to be Golgi membranes (Fig. 6). The sequence of events leading to assembly of viral particles is different for viruses in the Bunyaviridae as compared to those of other negative-strand RNA virus families, such as Rhabdoviridae, Orthomyxoviridae, and Paramyxoviridae. It has been demonstrated with these other viruses that their matrix (M) protein is responsible for bridging the gap between the integral viral envelope proteins and their nucleocapsids and acts as the nucleating step to signal assembly of virions at the cell surface (see Chapters 31, 34, and 39). Unlike these viruses, members of the Bunyaviridae family do not have an M protein. Instead, the early events of assembly include an interaction between viral ribonucleoprotein (RNP) structures, which accumulate on the cytoplasmic side of vesicular membranes, and viral envelope proteins, which are displayed on the luminal side (152) (see inset, Fig. 4). The viral RNP and spike structures have been observed only on the portion of the Golgi vesicle membrane directly involved in the budding process and not on adjacent areas of the same membrane. RNPs could not be found under membranes with no spikes, suggesting that some sort of transmembranal recognition between the viral glycoproteins and the N protein is prerequisite to budding (152). Candidate transmembrane regions have been predicted from hydropathic characteristics of derived amino acid sequences representing the envelope proteins of all members of the Bunyaviridae examined to date. Direct examination of the phlebovirus Karimabad, by enzymatic digestion of exposed proteins embedded in intracellular membranes, demonstrated that approximately 12% of G1 and/or G2 was exposed on the cytoplasmic face of membranes in infected cells and was accessible to digestion. A large protease-resistant fragment was identified, which was presumably sequestered in the membrane in a manner that rendered it safe from enzymatic digestion (152). These enzyme-resistant fragments may therefore represent transmembrane regions of proteins, which could provide the interaction between RNPs and the cellular membranes required for envelopment.

After the particles bud into the Golgi cisternae, it is believed that they are released in individual small vesicles in a manner analogous to secretory granules of other cell types (23,140,152). The release of virus from infected cells presumably occurs when the cytoplasmic, virus-containing vesicles fuse with the cellular plasma membrane, that is, via normal exocytosis.

Although morphogenesis in association with the Golgi appears to be a Bunyaviridae familial trait, it is not an absolute requirement for virion production. Studies on RVFV infection of primary rat hepatocytes demonstrated that mature virions can bud from the plasma membrane as well as into Golgi cisternae of the same cells (4). Because RVFV is a hepatotropic virus *in vivo* and can cause liver necrosis and death in animals, these results raise questions of possible differences in morphogenesis in target versus nontarget cell types that might affect pathogenesis and/or immune defense mechanisms.

## EFFECTS OF VIRAL REPLICATION ON HOST CELLS

The cytopathic effects observed in cultured cells infected with members of the Bunyaviridae vary widely, depending both on the virus and the type of host cell studied. Viruses in all genera except the *Hantavirus* genus are capable of alternately replicating in vertebrates and arthropods and generally are cytolytic for their vertebrate hosts but cause little or no cytopathogenicity in their invertebrate hosts (77,92). Some viruses display a very narrow host range, especially for arthropod vectors. Although the reason for this has not been defined completely, studies on LAC variant and revertant viruses suggested that the specificity was related to G1, probably at the level of viral attachment to susceptible cells (154). In natural infections of mammals, viruses are often targeted to a particular organ or cell type. For example, bunyaviruses such as LAC appear to be neurotropic (110), the phlebovirus RVFV

is primarily hepatotropic (4,5,37,121), and the hantavirus HTN remains persistent in rodent lungs (88). It will be interesting to determine whether this targeting is due solely to host-cell receptors or to other factors such as differences in effects on host-cell metabolism in targeted cell types versus the unnatural situation in cultured vertebrate cell lines.

### Effects on Host-Cell Metabolism

In vertebrate cells, bunyaviruses and phleboviruses have been shown to cause a reduction in host-cell protein synthesis, which decreases further as the infection progresses. A decline in BUN-infected BSC-1 host-cell protein synthesis was observed at 5 hr postinfection and by 7 hr was almost completely abolished (117). Similar results were obtained in LAC-infected BHK cells (93). RVFV-infected Vero cells displayed reduced host protein synthesis which gradually became more pronounced from 4 to 20 hr after infection (109).

No such reduction in host protein synthesis, even late in infection, has been observed in mammalian cells infected with the uukuvirus, UUK (122,158), or the nairovirus, Dugbe (27), both of which are transmitted by ticks rather than mosquitos. Host protein synthesis was somewhat inhibited, however, in a *Xenopus laevis* (frog) cell line infected with the nairovirus, Clo Mor, or the uukuvirus, St. Abb's head (163). Hantaviruses not only cause no detectable reduction in host macromolecular synthesis (38,143) but routinely establish persistent, noncytolytic infections in susceptible mammalian host cells, a finding consistent with their nonpathogenic persistence in their natural rodent hosts (88).

The arthropod-borne members of the Bunyaviridae, like most other arboviruses, cause little detectable cytopathology in mosquito cell cultures, and viral persistence is readily established (25,40,103,139). Unlike vertebrate cells, mosquito cells infected with the bunyavirus, Marituba, displayed no reduction in host macromolecular synthesis; thus, viral infection apparently does not drastically interfere with normal cellular processes (25). One suggested reason for this is that, in arthropod cells, excess viral proteins do not accumulate in the cells but rather are more efficiently processed into mature virions (103). In agreement with this, only N proteins of BUN and LAC could readily be detected in persistently infected mosquito cultures, although that protein was found to accumulate (40,139). Alternatively, it was suggested that the viral transcriptase may be less active in arthropod cells than in mammalian cells and that the endonuclease activity of the polymerase (which is used to acquire transcriptional primers) is detrimental to host-cell messages. A reduced level of activity of the viral transcriptase would therefore result in less damage to host-cell messages and consequently to protein synthesis (139).

Another possible explanation for persistence is the generation of defective or interfering viruses. Such an interfering BUN virus was recovered from persistently infected mosquito cells and effectively inhibited infection of normal mosquito cells by standard BUN (40). No evidence for deleted RNAs was obtained; thus, a persistence mechanism involving classical defective interfering particles could not be postulated (40,139). The exact nature of the interference remains to be determined.

## CONCLUSION

The Bunyaviridae family consists of a large and widely diverse group of viruses and has been divided into five genera based on serological relationships of members. Relatively few viruses in the family have been studied at the molecular level. The least characterized viruses in the Bunyaviridae are those in the *Nairovirus* genus, for which little replication information is available. Representatives of each genus have been found to display common morphological, biochemical, and genetic attributes yet have unique replicative properties and often extremely different biological characteristics. All viruses have three-segmented, single-stranded RNA genomes and, for those studied, encode their nucleocapsid and envelope proteins in the S and M genome segments, respectively. Completely different mechanisms for expression of the S genome segment products have been demonstrated in three of the five genera. Viruses in this family generally display an unusual Golgi-associated morphogenesis and usually acquire their envelopes by budding into intracytoplasmic vacuoles. The replication properties of members of the Bunyaviridae have only recently been elucidated and much remains to be learned, particularly with regard to the L genome segments and their presumed polymerase products.

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